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(57) Abstract

A method of modulating the development of a cutaneous appendage, consisting of modulating FGF-2 activity in one or both of the dermis and epidermis.

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MODULATING EPITHELIAL-MESENCHYMAL INTERACTIONS

BACKGROUND OF THE INVENTION

Epithelial-mesenchymal interactions play an essential role in the morphogenesis of cutaneous appendages (Sengel, P. Morphogenesis of Skin. Cambridge University Press, Cambridge, England (1976)) (feathers, scales and hairs), the limb (Fallon, J.F. Lopez, A. Ross, M.A., Savage, M.P., Olwin, B.O. and Simandl, B.K., *Science* 264:104-107 (1994),8,9), tooth (Vainio, S. Karavanova, I., Jowett, A. and Thesleff, I., *Cell* 75:45-58 (1993)), kidney (Patterson, L.T. and Dressler, G.R., *Curr. Opin. in Gen. and Dev.* 4:696-702 (1994)), lung (Peters, K., Werner, S., Liao X., Wert, S., Whitsett, J. and Williams, L. *EMBO J.* 13:3296-3201 (1994)) and mammary gland (Cuhna, G.R., *Cancer* 74:1030-1044 (1994)). In the feather or scale forming regions of the skin of the chick embryo, the epidermal placode is the first morphological prediction for the site of the formation of an appendage (Sengel, P. Morphogenesis of Skin. Cambridge University Press, Cambridge, England (1976)). Heterotypic and heterochronic recombinations of ectodermal and mesodermal components of skins indicate that the formation of the placodes is determined by mesodermal signals. Dermal signals also determine whether scales or feathers will develop, and, if feathers develop, their arrangement in a precise hexagonal pattern. The placodes in turn provide a signal which results in the formation of dermal condensations immediately beneath the placode (Sengel, P. Morphogenesis of Skin. Cambridge University Press, Cambridge, England (1976)). In feather forming regions, continued reciprocal interactions between the cells of the dermal condensation formation and those of the epidermal placode of each individual feather germ result in the outgrowth of the feather germ which at first is radially symmetrical and subsequently grows in a posterior direction. The anteroposterior direction of the outgrowth of the feather is determined by the ectoderm (Novel, G.J., *Embryol. Exp. Morph.* 30:605-633 (1973)). A number of studies have suggested that cell adhesion molecules, extracellular matrix molecules, and transcription factors may be involved in skin morphogenesis (Chuong, C-M., *BioEssays* 15:513-552 (1993), Noveen, A., Jiang, T-X, Ting-Berreth, S.A. and Chuong, C-M., *J. Invest. Derm.* 104:711-719 (1995)). However, no specific molecular mechanisms have been identified to date.

SUMMARY OF THE INVENTION

In general, the invention features, a method of modulating an epidermal-dermal interaction, or a process or product that results from an epidermal-dermal interaction.

Such interactions include, e.g., placode formation, dermal condensation formation, FGFR-1 induction, or the development of a cutaneous appendage, e.g., a hair, a scale, or a feather. The method includes: modulating, e.g., promoting or inhibiting, FGF-2 activity in one or both of the dermis and epidermis.

In preferred embodiments FGF-2 activity is promoted. FGF-2 activity can be promoted, e.g., by administration of exogenous FGF-2 or a fragment thereof, or a peptide or non-peptide analog thereof. FGF-2 activity can also be promoted by administering a nucleic acid which encodes FGF-2 or a fragment thereof, or a peptide analog thereof, or by promoting endogenous FGF-2 activity.

In preferred embodiments FGF-2 activity is inhibited. FGF-2 activity can be inhibited, e.g., by: administration of an FGF-2 binding molecule, e.g., an anti-FGF-2 antibody or FGF2-binding fragment thereof, or an FGF2-binding fragment of the FGF-2 receptor; administering a compound which inhibits, e.g., competitively or noncompetitively, the binding of FGF-2 to its receptor, e.g., a fragment of FGF-2 which binds the receptor but which lacks biological, e.g., signal transduction, activity, a peptide or non-peptide analog or a small molecule mimic of FGF-2 which binds the receptor but which lacks biological, e.g., signal transduction, activity; administering a nucleic acid which encodes an antisense molecule which inhibits FGF-2 expression.

In preferred embodiments the method is practiced *in vivo* and the subject is a vertebrate, e.g., a bird, or a mammal, e.g., a rodent, e.g., a mouse or a rat, or a primate, e.g., a nonhuman primate or a human. In preferred embodiments: the subject is other than a bird, a rodent, e.g., a mouse or a rat, or a nonhuman primate.

In preferred embodiments the method is performed *in vivo* and: the subject is wild type for one or more loci which condition the development of cutaneous appendages, e.g., the subject is wildtype for the scaleless locus; the subject is heterozygous, hemizygous, or homozygous, for a mutation at one or more loci which condition the development of cutaneous appendages, e.g., the subject carries one or more mutant alleles of the scaleless locus; the subject is a transgenic animal which is heterozygous, hemizygous, or homozygous, for a mutation at one or more loci which condition the development of cutaneous appendages, e.g., the subject carries one or more mutant alleles of the scaleless locus.

In preferred embodiments the method is practiced *in vitro*, e.g., on a tissue or cell, e.g., a cultured cell.

In preferred embodiments a compound which modulates FGF-2 activity is administered at least once and preferably at least 2, 4, or 10 times. The period between administrations can be more than 2, 4, 10 or 30 days but less than 1 year.

In preferred embodiments FGF-2 activity is modulated, e.g., increased or decreased, in the dermis.

In preferred embodiments one or both of the dermis or epidermis is: fetal dermis or epidermis; adult dermis or epidermis.

In another aspect, the invention features, a method of modulating the development of a cutaneous appendage, e.g., a hair, a scale, or a feather. The method includes: modulating, e.g., promoting or inhibiting, FGF-2 activity in one or both of the dermis and epidermis.

In preferred embodiments FGF-2 activity is promoted. FGF-2 activity can be promoted, e.g., by administration of exogenous FGF-2 or a fragment thereof, or a peptide or non-peptide analog thereof. FGF-2 activity can also be promoted by administering a nucleic acid which encodes FGF-2 or a fragment thereof, or a peptide analog thereof, or by promoting endogenous FGF-2 activity.

In preferred embodiments FGF-2 activity is inhibited. FGF-2 activity can be inhibited, e.g., by: administration of an FGF-2 binding molecule, e.g., an anti-FGF-2 antibody or FGF-2 binding fragment thereof, or an FGF-2 binding fragment of the FGF-2 receptor; administering a compound which inhibits, e.g., competitively or noncompetitively, the binding of FGF-2 to its receptor, e.g., a fragment of FGF-2 which binds the receptor but which lacks biological, e.g., signal transduction, activity, a peptide or non-peptide analog or a small molecule mimic of FGF-2 which binds the receptor but which lacks biological, e.g., signal transduction, activity; administering a nucleic acid which encodes an antisense molecule which inhibits FGF-2 expression.

In preferred embodiments the method is practiced *in vivo* and the subject is a vertebrate, e.g., a bird, or a mammal, e.g., a rodent, e.g., a mouse or a rat, or a primate, e.g., a nonhuman primate or a human. In preferred embodiments: the subject is other than any of a bird, a rodent, e.g., a mouse or a rat, or a nonhuman primate.

In preferred embodiments the method is performed *in vivo* and: the subject is wild type for one or more loci which condition the development of cutaneous appendages, e.g., the subject is wildtype for the scaleless locus; the subject is heterozygous, hemizygous, or homozygous, for a mutation at one or more loci which condition the development of cutaneous appendages, e.g., the subject carries one or more mutant alleles of the scaleless locus; the subject is a transgenic animal which is heterozygous, hemizygous, or homozygous, for a mutation at one or more loci which condition the development of cutaneous appendages, e.g., the subject carries one or more mutant alleles of the scaleless locus.

In preferred embodiments the method is practiced *in vitro*, e.g., on a tissue or cell, e.g., a cultured cell.

In preferred embodiments a compound which modulates FGF-2 activity is administered at least once and preferably at least 2, 4, or 10 times. The period between administrations can be more than 2, 4, 10 or 30 days but less than 1 year.

In preferred embodiments FGF-2 activity is modulated, e.g., increased or decreased, in the dermis.

In preferred embodiments one or both of the dermis or epidermis is: fetal dermis or epidermis; adult dermis or epidermis.

In another aspect, the invention features, a method for promoting development of a cutaneous appendage, e.g., a hair, a scale, or a feather, in a tissue or subject, the method including administering a therapeutically effective amount of exogenous FGF-2 or fragment thereof to the tissue or subject.

In preferred embodiments, the subject is a vertebrate, e.g., a bird, or a mammal, e.g., a rodent, e.g., a mouse or a rat, or a primate, e.g., a nonhuman primate or a human. In preferred embodiments: the subject is other than any of a bird, a rodent, e.g., a mouse or a rat, or a nonhuman primate.

In preferred embodiments, the exogenous FGF-2 or a fragment thereof is provided to a subject preferably directly, e.g., locally, as by injection or topical administration to a given site, or systemically, e.g., parenterally or orally.

In another aspect, the invention features, a method of evaluating a compound for the ability to bind an FGF-2 polypeptide and the ability to modulate an FGF-2/FGFR-1 interaction or otherwise modulate FGF-2 activity. The compound can be, e.g., a polypeptide or non-peptide compound, e.g., a naturally occurring ligand of an FGF-2 polypeptide, e.g., an FGFR-1 polypeptide, e.g., a fragment of an FGFR-1 polypeptide. The method includes: contacting the compound with the FGF-2 polypeptide; and evaluating the ability of the compound to interact with, e.g., to bind or form a complex with the FGF-2 polypeptide, e.g., the ability of the compound to inhibit an FGF-2/FGFR-1 interaction. This method can be performed in vitro, e.g., in a cell free system, or in vivo, e.g., in a two-hybrid interaction trap assay. This method can be used to identify compounds, e.g., fragments or analogs of FGFR-1, which are agonists or antagonists of FGF-2. The method can further include the step of applying the evaluated compound to an animal, tissue, or cell to further evaluate its effect on an epidermal-dermal interaction, or a process or product that results from an epidermal-dermal interaction, e.g., placode formation, dermal condensation formation. FGFR-1 induction, or the development of a cutaneous appendage, e.g., a hair, a scale, or a feather.

In another aspect, the invention features, a method of evaluating a first compound, e.g., an FGF-2 polypeptide, for the ability to bind a second compound, e.g., a second polypeptide or non-peptide compound, e.g., a naturally occurring ligand of FGF-2 polypeptide, e.g., an FGFR-1, or a fragment thereof. The method includes: contacting the first compound with the second compound; and evaluating the ability of the first compound to form a complex with the second compound. This method can be performed in vitro, e.g., in a cell free system, or in vivo, e.g., in a two-hybrid interaction trap assay. This method can be used to identify compounds, e.g., fragments or analogs of FGF-2, which are agonists or antagonists of FGF-2. This method can be used to identify compounds, e.g., fragments or analogs of FGFR-1, which are agonists or antagonists of FGF-2. The method can further include the step of applying the evaluated compound to an animal, tissue, or cell to further evaluate its effect on an epidermal-dermal interaction, or a process or product that results from an epidermal-dermal interaction, e.g., placode formation, dermal condensation formation, FGFR-1 induction, or the development of a cutaneous appendage, e.g., a hair, a scale, or a feather.

In yet another aspect, the invention features a method for evaluating a compound, e.g., for the ability to modulate an interaction, e.g., the ability to inhibit an interaction of an FGF-2 polypeptide, with a second polypeptide, e.g., a polypeptide, e.g., a natural ligand of the FGF-2 polypeptide, e.g., an FGFR-1, or a fragment thereof. The method includes the steps of (i) combining the second polypeptide (or preferably a purified preparation thereof), an FGF-2 polypeptide, (or preferably a purified preparation thereof), and a compound, e.g., under conditions wherein in the absence of the compound, the second polypeptide, and the FGF-2 polypeptide, are able to interact, e.g., to bind or form a complex; and (ii) detecting the interaction, e.g., detecting the formation (or dissolution) of a complex which includes the second polypeptide, and the FGF-2 polypeptide. A change, e.g., a decrease or increase, in the formation of the complex in the presence of a compound (relative to what is seen in the absence of the compound) is indicative of a modulation, e.g., an inhibition or promotion, of the interaction between the second polypeptide, and the FGF-2 polypeptide. In preferred embodiments: the second polypeptide, and the FGF-2 polypeptide, are combined in a cell-free system and contacted with the compound; the cell-free system is selected from a group consisting of a cell lysate and a reconstituted protein mixture; the FGF-2 polypeptide, and the second polypeptide are simultaneously expressed in a cell, and the cell is contacted with the compound, e.g. in an interaction trap assay (e.g., a two-hybrid assay). This method can be used to identify compounds, e.g., fragments or analogs of FGFR-1, which are agonists or antagonists of FGF-2. The method can further include the step of applying

the evaluated compound to an animal, tissue, or cell to further evaluate its effect on an epidermal-dermal interaction, or a process or product that results from an epidermal-dermal interaction, e.g., placode formation, dermal condensation formation, FGFR-1 induction, or the development of a cutaneous appendage, e.g., a hair, a scale, or a feather.

A molecule has FGF-2 biological activity if it has one or more of the following properties: (1) it induces an epidermal-dermal interaction, or process or product that results from an epidermal-dermal interaction, e.g., placode formation, dermal condensation formation, FGFR-1 induction, or the development of a cutaneous appendage, e.g., a hair, a scale, or a feather, when applied to sc/sc chick tissue as described herein; (2) it is mitogenic; (3) it binds to the FGFR-1 receptor; (4) it is an antagonist or agonist of one or more of the properties described above.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DETAILED DESCRIPTION

FGF-2 and Morphological Development

The development of epidermal appendages such as feathers, scales and hairs results from a series of reciprocal interactions between the ectoderm and the mesoderm

of the embryonic skin (Sengel, P. Morphogenesis of Skin. Cambridge University Press, Cambridge, England (1976)). At the morphological level these interactions are first reflected in the appearance of ectodermal placodes and then by the formation of condensations in the dermis immediately beneath the placodes. The absence of feathers and scales in the scaleless (sc/sc) mutant (Abbott, U.K., and Asmundson, V.S., *J. Hered.* 18:63-67 (1957)) results from an ectodermal defect (Goetinck, P.F. and Abbott, U.K., *J. Exp. Zool.* 154:7-19 (1963), Sengel, P. and Abbott, U.K., *J. Hered.* 54:254-262 (1963), Song, H.-K. and Sawyer, R.H., Dev. Dyn. In Press (1995)) and such embryos fail to develop ectodermal placodes in their skin (Goetinck, P.F. and Sekellick, M.J., *Dev. Biol.* 28:636-648 (1972)). The role of FGF-2 in the epithelial-mesenchymal signaling during the initiation of the development of feathers was examined. Here a spatially and temporally restricted pattern of transcription for the genes that encode FGF-2 and FGFR-1 in skins of normal embryos is reported. FGF-2 expression is restricted to the epidermal placodes whereas FGFR-1 expression is limited to the dermal condensations. Transcription of these genes could not be detected in sc/sc skins. Treatment of sc/sc skins with FGF-2 results in the formation of feathers at the site of application of the growth factor. Furthermore, FGFR-1 transcripts are evident in the mesoderm of the feathers induced in the treated mutant skins. Thus, FGF-2 has been identified as an early ectodermal signal in the epithelial-mesenchymal interactions that occur during feather development. The observation that this growth factor can rescue the mutant phenotype of sc/sc embryos that lack epidermal placodes suggests that FGF-2 is, or is downstream from, the signal that the mutant ectoderm fails to generate.

Transcription of FGF-2

To examine the role of FGF-2 in feather development the spatial and temporal pattern of transcription of the FGF-2 gene (Zuniga, A. Mejia, B., Meijers, C. and Zeller, R. *Dev. Biol.* 157:110-118 (1993)) was examined by whole mount in situ hybridization. In stage 33 (Hamburger, V. and Hamilton, H.L., *J. Morphol.* 88:49-92 (1951)) (E8) skins of normal embryos, FGF-2 transcripts could be detected in all feather germs. The staining of early feather germs appears as full circles arranged in a hexagonal pattern. In the spinal tract, FGF-2 mRNA could be detected in the feather germs at the very edge of the tract where the formation of epidermal placodes precedes the formation of dermal condensations (Sengel, P. Morphogenesis of Skin. Cambridge University Press, Cambridge, England (1976)). Cross sections of stained embryos indicate that FGF-2 mRNA expression is restricted to the epidermal placodes of the feather germs. Thus, spatially FGF-2 expression is restricted to the epidermal placodes and temporally FGF-2 expression precedes the formation of the dermal condensations. In more developed

feather buds, FGF-2 mRNA was seen in the distal part of the elongating buds. As feather buds grow out into feather filaments, the entire length of the filament expresses FGF-2. No FGF-2 mRNA could be detected in the bases of the filaments. In all stages examined, FGF-2 was absent from the interbud or interfeather regions.

No transcripts for FGF-2 could be detected in the skins of sc/sc embryos of comparable stages of development. On rare occasions, sc/sc embryos develop a few feathers. In these exceptional feathers, it was difficult to determine if FGF-2 is expressed because the hybridization signal could not be visualized in the highly pigmented mutant feathers.

Whole-mount in situ hybridizations were performed essentially as described in Riddle, R.D., Johnson, R.L., Laufer, E. and Tabin, C., *Cell* 75:1401-1416 (1993). FGF-2 RNA probe was made using the full-length cDNA for alt-FGF-2 (Zuniga, A. Mejia, B., Meijers, C. and Zeller, R. *Dev. Biol.* 157:110-118 (1993)).

Ectoderm signaling involves FGF-2

Since FGF-2 transcripts are restricted to the ectodermal placodes of normal feather germs and these structures are absent from sc/sc skins, it was hypothesized that the defective signaling in the mutant ectoderm may involve FGF-2. To test this hypothesis, exogenous FGF-2 was applied to skins obtained from the dorsal tract of eight day old sc/sc embryos. Heparin beads loaded with FGF-2 were placed on skins supported on millipore filer chambers and cultured on the chorioallantoic membrane of 10 day old chick embryos for three or five days.

After three days of culture, feather buds are clearly evident in the mutant skins where the beads are deposited. In many cases, feather buds developed all around the beads. Table 1 indicates the correlation between the sites of bead deposition and the induction of feather buds. Eighty-three percent of feather buds observed in the center of the skins were correlated with the presence of beads. On the lateral side of the explants the correlation between bead location and feather development was 95%. The difference between sites may reflect the occasional development of a few feathers in intact sc/sc skin along the center of the dorsal feather tract. Such spurious feather development is never observed in the lateral side of the dorsal tract of sc/sc skin *in vivo*. PBS soaked beads, used as control, did not stimulate any feather development in the sc/sc skins. No hexagonal pattern in the arrangement of the feathers was observed. Most of the feather germs were highly pigmented and they were variable in size and shape.

TABLE 1

Spatial correlation of site of feather formation with the location of the FGF-2 beads.

	feather buds correlated with beads	feather buds not correlated with beads
central region	83% (89 buds)	17% (18 buds)
lateral region	95% (36 buds)	5% (4 buds)

-- A total of seven skins were examined after 3 days of culture on the chorioallantoic membrane.

Cross sections of feathers in skins cultured for 5 days show normally arranged barb ridges and barbule cells indicating that they are normally developed feathers. The length of the feathers is relatively uniform but their size and shape can be irregular. Such irregularities are never observed in normal feathers. Most of the abnormal feathers seem to result from fusion of feather buds that can be seen at three days to be located close to each other. Fusions can occur such that the feather filaments have separate bases or tips. The latter type of fusion can result in fork-like structures. Another form of abnormal filament involves fusions in the middle of two filaments with separate bases and tips. A cross sectional view of one of these fused feathers indicates that the fused feathers are outlined by one feather sheath but a septum divides the structures inside into two separate groups. Serial sections indicated the presence of separate follicles. Thus, FGF-2 which is expressed in the epidermal placode of feather germs in normal embryos can induce the outgrowth of feathers in sc/sc skins that lack epidermal placodes. The identification of FGF-2 as an ectodermal signal in feather development is similar to its role n the limb where FGF-2 and FGF-4 can substitute for the apical ectodermal ridge in limb outgrowth (Fallon, J.F. Lopez, A. Ross, M.A., Savage, M.P., Olwin, B.O. and Simandl, B.K., *Science* 264:104-107 (1994), Niswander, L., Ticle, C., Vogel, A., Booth, I. and Martin G.R., *Cell* 75:579-587 (1993)).

The orientation of the feathers induced in the mutant skins was random. Thus the uniform antero-posterior orientation of feathers in normal embryos is not seen in the feathers induced in sc/sc skins by FGF-2. Since antero-posterior orientation of feather germs is determined by the ectoderm (Novel, G.J., *Embryol. Exp. Morph.* 30:605-633 (1973)), the failure of the induced sc/sc feathers to orient posteriorly may also result from the mutant ectodermal defect which is independent of FGF-2.

The experiments were performed essentially as follows. Heparin beads (Bio-Rad, Hercules, California) were soaked in solution of FGF-2 (1mg/ml PBS, Bovine FGF basic, R&D Systems, Minneapolis, Minnesota) for two hours, washed three times in

PBS and applied onto the surface of the sc/sc skins. The culturing of the skins on the chorioallantoic membrane was done as previously described (Noveen, A., Jiang, T-X, Ting-Berreth, S.A. and Chuong, C-M., *J. Invest. Derm.* 104:711-719 (1995)).

Expression of FGFR-1

The pattern of transcription of FGFR-1 (Pasquale, E.G. and Singer, *J.S. Proc. Natl. Acad. Sci.*, USA 86:5449-5454 (1989)) was also examined in developing skins. Transcripts for the FGFR-1 could be detected in the feather germs and outgrowing feather filaments in normal embryos. The expression of FGFR-1 was restricted to the dermal condensation formation of the feathers. These observations confirm those of others (Nopji, S., Koyama E., Myokai, F., Nohno, T., Ohuchi, H., Nishikawa, K. and Taniguchi, S., *Progress in Clin. and Biol. Res.* 383B:645-654 (1993)). In contrast, no mRNA for FGFR-1 could be detected in skins of sc/sc embryos. When feathers are induced by FGF-2 in sc/sc skins, FGFR-1 transcripts can be detected in the dermis of the outgrowing feather. Since the FGFR-1 gene is not transcribed in intact and untreated sc/sc skin, this observation suggests that the response to FGF-2 induction involves the activation of the FGFR-1 gene in the dermis. The induction of receptors by ligands has also been reported for the activin receptors cActR-IIB (Stern, C.D., Yu, R.T., Kakizuka, A., Kintner, C.R., Mathews, L.S., Vale, W. W., Evans, R.M. and Umesono, K. *Dev. Biol.* 172:192-205 (1995)) and cActR-IIa (Levin, M., Johnson, R.L., Stern, C.D., Kuehn, M. and Tabin, C., *Cell* 82:803-814 (1995)) by the application of exogenous activin.

Whole-mount in situ hybridizations were essentially performed as describe in Riddle, R.D., Johnson, R.L., Laufer, E. and Tabin, C., *Cell* 75:1401-1416 (1993). FGFR-1 probe was derived from a chick cDNA clone (Pasquale, E.G. and Singer, *J.S. Proc. Natl. Acad. Sci.*, USA 86:5449-5454 (1989)). The RNA probe was a 490 bp fragment encoding the 5' end and the first immunoglobulin-like domain of FGFR-1.

Induction of Feathers by FGF-2

The induction of feathers in sc/sc skin by exogenous FGF-2 is dependent on the developmental stage of the mutant skins. Feathers could be induced by FGF-2 in sc/sc skins from seven and eight day old embryos but not from eleven day old embryos. These results are similar to those obtained from tissue recombination experiments in which sc/sc dermis of different stages was recombined with normal eleven day foot ectoderm (Noveen, A., Jiang, T-X, Ting-Berreth, S.A. and Chuong, C-M., *J. Invest. Derm.* 104:711-719 (1995)). Feathers develop when such foot ectoderm is recombined with dorsal mesoderm (Rawles, M.E., *J. Embryol. Exp. Morph.* 11:765-789 (1963)). Eight day sc/sc dermis can participate with normal ectoderm in feather formation, but this ability is gradually lost from mutant mesoderm of increasing developmental ages

(Noveen, A., Jiang, T-X, Ting-Berreth, S.A. and Chuong, C-M., *J. Invest. Derm.* 104:711-719 (1995)). These results indicate that there is a developmental window in which the mutant mesoderm can respond to both FGF-2 signaling and to cues from normal ectoderm and they are consistent with the suggestion that epidermal placode derived FGF-2 is an important signal in normal feather development and that this signal is absent from the skin of the sc/sc mutant that lacks epidermal placodes.

Skin culture and bead preparation were performed as described in the example above.

In the present study the role of FGF-2 in the epithelial-mesenchymal signaling during the initiation of feather germ formation in genetically normal and scaleless embryos was examined. Feathers and scales do not develop in embryos that are homozygous for the recessive gene scaleless (Abbott, U.K., and Asmundson, V.S., *J. Hered.* 18:63-67 (1957)). The skins of these embryos do not develop ectodermal placodes (Goetinck, P.F. and Sekellick, M.J., *Dev. Biol.* 28:636-648 (1972)). Thus, the scaleless mutation affects the ectoderm of the mutant skins in such a way that the normal sequence of tissue interactions is interrupted. It is not clear if an early dermal signal cannot be received by the ectoderm or if the ectoderm cannot respond to that signal. The mutant mesoderm is fully capable of participating in feather and scale development when recombined with genetically normal ectoderm (Goetinck, P.F. and Abbott, U.K., *J. Exp. Zool.* 154:7-19 (1963), Sengel, P. and Abbott, U.K., *J. Hered.* 54:254-262 (1963), Song, H.-K. and Sawyer, R.H., Dev. Dyn. In Press (1995)).

FGF-2 signaling is sufficient to form dermal condensations and this signaling is direct

There is a spatially and temporally restricted pattern of transcription for the genes that encode fibroblast growth factor (FGF)-2 and fibroblast growth factor receptor (FGFR)-1 in developing feather germs of the genetically normal chick embryo. FGF-2 expression is restricted to the epidermal placodes whereas FGFR-1 expression is limited to the dermal condensations. Transcription of these genes could not be detected in skins of mutant embryos that are homozygous for the recessive gene scaleless (sc/sc). Such mutant embryos fail to develop feathers as a result of an ectodermal defect. Treatment of sc/sc skins with FGF-2 results in the formation of feathers at the site of application of the growth factor and the induced feathers express FGFR-1 in their dermal condensations. From these the role of FGF-2 as an epidermal signal in early feather germ formation was established. The observation that FGF-2 can rescue the mutant phenotype of sc/sc embryos suggests that FGF-2 either is, or is downstream, from the signal that the sc/sc mutant ectoderm fails to generate. The rescue data could be interpreted to mean either that the FGF-2 signal acts directly on the mesoderm to form

dermal condensations or, alternatively, that the FGF-2 treatment initiates a series of reciprocal interactions between the mesoderm and the ectoderm before the ectoderm instructs the mesoderm to form dermal condensations.

To test the hypothesis that an FGF-2 signal is sufficient to form dermal condensations in the underlying mesoderm, denuded mutant mesoderm was exposed to FGF-2. This mesoderm is considered to be both competent and naive in that it can participate in feather development when combined with genetically normal ectoderm but it has not been exposed to any ectodermal signals since the mutant ectoderm with which it has been associated is defective. When FGF-2 beads were placed on denuded mutant mesoderm the formation of dermal condensations in the mesoderm was observed. These condensations are positive for FGFR-1 and for BMP-2, two molecular markers that are normally expressed by cells that make up the dermal condensations. Thus, FGF-2 signaling is sufficient to form dermal condensations and this signaling is direct.

Formulations and Methods for Administering FGF-2

The FGF-2, FGF-2 fragment or analog may be provided to a subject by any suitable means, preferably directly (e.g., locally, as by injection or topical administration to a tissue locus) or systemically (e.g., parenterally or orally). Where the compound of the invention is to be provided parenterally, such as by intravenous, subcutaneous, intramuscular, or by aerosol administration, the compound of the invention preferably comprises part of an aqueous solution. The solution is physiologically acceptable so that in addition to delivery of the desired compound of the invention to the subject, the solution does not otherwise adversely affect the subjects' electrolyte and volume balance. The aqueous medium for the FGF-2 or a fragment thereof thus may comprise normal physiologic saline (9.85% NaCl, 0.15M), pH 7-7.4).

Useful solutions for parenteral administration may be prepared by any of the methods well known in the pharmaceutical art, described, for example, in Remington's Pharmaceutical Science (Gennaro, A., ed.), Mack Pub., 1990. Formulations may include, for example, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Formulations for direct administration, in particular, may include glycerol and other compositions of high viscosity to help maintain the compound of the invention at the desired locus. Biocompatible, preferably bioresorbable, polymers, including for example, hyaluronic acid, collagen, tricalcium phosphate, polybutyrate, lactide and glycolide polymers, and lactide/glycolide copolymers, may be useful excipients to control the release of the compound of the invention *in vivo*. Other potentially useful parenteral delivery systems for the compound of the invention include ethylenevinyl acetate copolymer particles, osmotic pumps.

implantable infusion system. and liposome. Formulations for inhalation administration contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9- laurly ether, glycocholate and deoxycholate.

Formulations for topical administration to the skin surface may be prepared by dispersing the compound of the invention with a dermally acceptable carrier such as a lotion, cream, ointment or soap. Particularly useful are carriers capable of forming a film or layer over the skin to localize application and inhibit removal.

Production of Fragments and Analogs

The inventor has discovered that an epidermal-dermal interaction can be modulated by modulating, e.g., promoting or inhibiting, the activity of FGF-2. Once this showing was made, one skilled in the art can alter the FGF-2 structure, e.g., by producing fragments or analogs, and test the newly produced structures for activity. Examples of prior art methods which allow the production and testing of fragments and analogs are discussed below. These, or analogous methods, can be used to make and screen fragments and analogs of an FGF-2 polypeptide, which bind FGFR-1. Likewise these methods can be used to make fragments and analogs of FGF-2 polypeptide ligands, e.g., FGFR-1, which bind an FGF-2 polypeptide.

Generation of Fragments

Fragments of a protein can be produced in several ways, e.g., recombinantly, by proteolytic digestion, or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end (for a terminal fragment) or both ends (for an internal fragment) of a nucleic acid which encodes the polypeptide. Expression of the mutagenized DNA produces polypeptide fragments. Digestion with "end-nibbling" endonucleases can thus generate DNA's which encode an array of fragments. DNA's which encode fragments of a protein can also be generated by random shearing, restriction digestion or a combination of the above-discussed methods.

Fragments can also be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, peptides of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or divided into overlapping fragments of a desired length.

Generations of Analogs: Production of Altered DNA and Peptide Sequences by Random Methods

Amino acid sequence variants of a protein can be prepared by random mutagenesis of DNA which encodes a protein or a particular domain or region of a

protein. Useful methods include PCR mutagenesis and saturation mutagenesis. A library of random amino acid sequence variants can also be generated by the synthesis of a set of degenerate oligonucleotide sequences. (Methods for screening proteins in a library of variants are elsewhere herein.)

PCR Mutagenesis

In PCR mutagenesis, reduced Taq polymerase fidelity is used to introduce random mutations into a cloned fragment of DNA (Leung et al., 1989, *Technique* 1:11-15). This is a very powerful and relatively rapid method of introducing random mutations. The DNA region to be mutagenized is amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, e.g., by using a dGTP/dATP ratio of five and adding Mn^{2+} to the PCR reaction. The pool of amplified DNA fragments are inserted into appropriate cloning vectors to provide random mutant libraries.

Saturation Mutagenesis

Saturation mutagenesis allows for the rapid introduction of a large number of single base substitutions into cloned DNA fragments (Mayers et al., 1985, *Science* 229:242). This technique includes generation of mutations, e.g., by chemical treatment or irradiation of single-stranded DNA *in vitro*, and synthesis of a complimentary DNA strand. The mutation frequency can be modulated by modulating the severity of the treatment, and essentially all possible base substitutions can be obtained. Because this procedure does not involve a genetic selection for mutant fragments both neutral substitutions, as well as those that alter function, are obtained. The distribution of point mutations is not biased toward conserved sequence elements.

Degenerate Oligonucleotides

A library of homologs can also be generated from a set of degenerate oligonucleotide sequences. Chemical synthesis of a degenerate sequences can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier 273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

Generation of Analogs: Production of Altered DNA and Peptide Sequences by Directed Mutagenesis

Non-random or directed, mutagenesis techniques can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

Alanine Scanning Mutagenesis

Alanine scanning mutagenesis is a useful method for identification of certain residues or regions of the desired protein that are preferred locations or domains for mutagenesis, Cunningham and Wells (*Science* 244:1081-1085, 1989). In alanine scanning, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine). Replacement of an amino acid can affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions are then refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed desired protein subunit variants are screened for the optimal combination of desired activity.

Oligonucleotide-Mediated Mutagenesis

Oligonucleotide-mediated mutagenesis is a useful method for preparing substitution, deletion, and insertion variants of DNA, see, e.g., Adelman et al., (*DNA* 2:183, 1983). Briefly, the desired DNA is altered by hybridizing an oligonucleotide encoding a mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the desired protein. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the desired protein DNA. Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely

complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci.* USA, 75: 5765[1978]).

Cassette Mutagenesis

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene*, 34:315[1985]). The starting material is a plasmid (or other vector) which includes the protein subunit DNA to be mutated. The codon(s) in the protein subunit DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the desired protein subunit DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are comparable with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated desired protein subunit DNA sequence.

Combinatorial Mutagenesis

Combinatorial mutagenesis can also be used to generate mutants. E.g., the amino acid sequences for a group of homologs or other related proteins are aligned, preferably to promote the highest homology possible. All of the amino acids which appear at a given position of the aligned sequences can be selected to create a degenerate set of combinatorial sequences. The variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For example, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential sequences are expressible as individual peptides, or alternatively, as a set of larger fusion proteins containing the set of degenerate sequences.

Primary High-Through-Put Methods for Screening Libraries of Peptide Fragments or Homologs

Various techniques are known in the art for screening generated mutant gene products. Techniques for screening large gene libraries often include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the genes under conditions in which detection of a desired activity, e.g., in this case, binding to FGF-2 or FGFR-1, facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the techniques described below is amenable to high through-put analysis for screening large numbers of sequences created, e.g., by random mutagenesis techniques.

Two Hybrid Systems

Two hybrid assays such as the system described above (as with the other screening methods described herein), can be used to identify fragments or analogs of an FGF-2 polypeptide which binds to the intracellular domain of FGFR-1. These may include agonists, superagonists, and antagonists. (The FGFR-1 domain is used as the bait protein and the library of variants of the FGF-2 are expressed as fish fusion proteins.) In an analogous fashion, a two hybrid assay (as with the other screening methods described herein), can be used to find fragments and analogs of FGFR-1 which bind an FGF-2 polypeptide.

Display Libraries

In one approach to screening assays, the candidate peptides are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to bind an appropriate receptor protein via the displayed product is detected in a "panning assay". For example, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140). In a similar fashion, a detectably labeled ligand can be used to score for potentially functional peptide homologs. Fluorescently labeled ligands, e.g., receptors, can be used to detect homolog which retain ligand-binding activity. The use of fluorescently labeled ligands, allows cells to be visually inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, to be separated by a fluorescence-activated cell sorter.

A gene library can be expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at concentrations

well over 10^{13} phage per milliliter, a large number of phage can be screened at one time. Second, since each infectious phage displays a gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd., and f1 are most often used in phage display libraries. Either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle. Foreign epitopes can be expressed at the NH_2-terminal end of pIII and phage bearing such epitopes recovered from a large excess of phage lacking this epitope (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) *J. Biol. Chem.* 267:16007-16010; Griffiths et al. (1993) *EMBO J* 12:725-734; Clackson et al. (1991) *Nature* 352:624-628; and Barbas et al. (1992) *PNAS* 89:4457-4461).

A common approach uses the maltose receptor of *E. coli* (the outer membrane protein, LamB) as a peptide fusion partner (Charbit et al. (1986) *EMBO* 5, 3029-3037). Oligonucleotides have been inserted into plasmids encoding the LamB gene to produce peptides fused into one of the extracellular loops of the protein. These peptides are available for binding to ligands, e.g., to antibodies, and can elicit an immune response when the cells are administered to animals. Other cell surface proteins, e.g., OmpA (Schorr et al. (1991) *Vaccines 91*, pp. 387-392), PhoE (Agterberg, et al. (1990) *Gene* 88, 37-45), and PAL (Fuchs et al. (1991) *Bio/Tech* 9, 1369-1372), as well as large bacterial surface structures have served as vehicles for peptide display. Peptides can be fused to pilin, a protein which polymerizes to form the pilus-a conduit for interbacterial exchange of genetic information (Thiry et al. (1989) *Appl. Environ. Microbiol.* 55, 984-993). Because of its role in interacting with other cells, the pilus provides a useful support for the presentation of peptides to the extracellular environment. Another large surface structure used for peptide display is the bacterial motive organ, the flagellum. Fusion of peptides to the subunit protein flagellin offers a dense array of may peptides copies on the host cells (Kuwajima et al. (1988) *Bio/Tech.* 6, 1080-1083). Surface proteins of other bacterial species have also served as peptide fusion partners. Examples include the *Staphylococcus* protein A and the outer membrane protease IgA of *Neisseria* (Hansson et al. (1992) *J. Bacteriol.* 174, 4239-4245 and Klauser et al. (1990) *EMBO J.* 9, 1991-1999).

In the filamentous phage systems and the LamB system described above, the physical link between the peptide and its encoding DNA occurs by the containment of the DNA within a particle (cell or phage) that carries the peptide on its surface. Capturing the peptide captures the particle and the DNA within. An alternative scheme

uses the DNA-binding protein LacI to form a link between peptide and DNA (Cull *et al.* (1992) *PNAS USA* 89:1865-1869). This system uses a plasmid containing the LacI gene with an oligonucleotide cloning site at its 3'-end. Under the controlled induction by arabinose, a LacI-peptide fusion protein is produced. This fusion retains the natural ability of LacI to bind to a short DNA sequence known as LacO operator (LacO). By installing two copies of LacO on the expression plasmid, the LacI-peptide fusion binds tightly to the plasmid that encoded it. Because the plasmids in each cell contain only a single oligonucleotide sequence and each cell expresses only a single peptide sequence, the peptides become specifically and stably associated with the DNA sequence that directed its synthesis. The cells of the library are gently lysed and the peptide-DNA complexes are exposed to a matrix of immobilized receptor to recover the complexes containing active peptides. The associated plasmid DNA is then reintroduced into cells for amplification and DNA sequencing to determine the identity of the peptide ligands. As a demonstration of the practical utility of the method, a large random library of dodecapeptides was made and selected on a monoclonal antibody raised against the opioid peptide dynorphin B. A cohort of peptides was recovered, all related by a consensus sequence corresponding to a six-residue portion of dynorphin B. (Cull et al. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89-1869)

This scheme, sometimes referred to as peptides-on-plasmids, differs in two important ways from the phage display methods. First, the peptides are attached to the C-terminus of the fusion protein, resulting in the display of the library members as peptides having free carboxy termini. Both of the filamentous phage coat proteins, pIII and pVIII, are anchored to the phage through their C-termini, and the guest peptides are placed into the outward-extending N-terminal domains. In some designs, the phage-displayed peptides are presented right at the amino terminus of the fusion protein. (Cwirla, et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 6378-6382) A second difference is the set of biological biases affecting the population of peptides actually present in the libraries. The LacI fusion molecules are confined to the cytoplasm of the host cells. The phage coat fusions are exposed briefly to the cytoplasm during translation but are rapidly secreted through the inner membrane into the periplasmic compartment, remaining anchored in the membrane by their C-terminal hydrophobic domains, with the N-termini, containing the peptides, protruding into the periplasm while awaiting assembly into phage particles. The peptides in the LacI and phage libraries may differ significantly as a result of their exposure to different proteolytic activities. The phage coat proteins require transport across the inner membrane and signal peptidase processing as a prelude to incorporation into phage. Certain peptides exert a deleterious

effect on these processes and are underrepresented in the libraries (Gallop et al. (1994) *J. Med. Chem.* 37(9):1233-1251). These particular biases are not a factor in the LacI display system.

The number of small peptides available in recombinant random libraries is enormous. Libraries of 10^7-10^9 independent clones are routinely prepared. Libraries as large as 10^{11} recombinants have been created, but this size approaches the practical limit for clone libraries. This limitation in library size occurs at the step of transforming the DNA containing randomized segments into the host bacterial cells. To circumvent this limitation, an *in vitro* system based on the display of nascent peptides in polysome complexes has recently been developed. This display library method has the potential of producing libraries 3-6 orders of magnitude larger than the currently available phage/phagemid or plasmid libraries. Furthermore, the construction of the libraries, expression of the peptides, and screening, is done in an entirely cell-free format.

In one application of this method (Gallop et al. (1994) *J. Med. Chem.* 37(9):1233-1251), a molecular DNA library encoding 10^{12} decapeptides was constructed and the library expressed in an *E. coli* S30 *in vitro* coupled transcription/translation system. Conditions were chosen to stall the ribosomes on the mRNA, causing the accumulation of a substantial proportion of the RNA in polysomes and yielding complexes containing nascent peptides still linked to their encoding RNA. The polysomes are sufficiently robust to be affinity purified on immobilized receptors in much the same way as the more conventional recombinant peptide display libraries are screened. RNA from the bound complexes is recovered, converted to cDNA, and amplified by PCR to produce a template for the next round of synthesis and screening. The polysome display method can be coupled to the phage display system. Following several rounds of screening, cDNA from the enriched pool of polysomes was cloned into a phagemid vector. This vector serves as both a peptide expression vector, displaying peptides fused to the coat proteins, and as a DNA sequencing vector for peptide identification. By expressing the polysome-derived peptides on phage, one can either continue the affinity selection procedure in this format or assay the peptides on individual clones for binding activity in a phage ELISA, or for binding specificity in a completion phage ELISA (Barret, et al. (1992) *Anal. Biochem* 204,357-364). To identify the sequences of the active peptides one sequences the DNA produced by the phagemid host.

Secondary Screens

The high through-put assays described above can be followed by secondary screens in order to identify further biological activities which will, e.g., allow one skilled

in the art to differentiate agonists from antagonists. The type of a secondary screen used will depend on the desired activity that needs to be tested. For example, an assay can be developed in which the ability to inhibit an interaction between a protein of interest and its respective ligand can be used to identify antagonists from a group of peptide fragments isolated though one of the primary screens described above.

Therefore, methods for generating fragments and analogs and testing them for activity are known in the art. Once the core sequence of interest is identified, it is routine to perform for one skilled in the art to obtain analogs and fragments.

Peptide Mimetics

The invention also provides for reduction of the protein binding domains of the FGF-2 polypeptides to generate mimetics, e.g. peptide or non-peptide agents. The peptide mimetics are able to disrupt binding of an FGF-2 to it's counter ligand, e.g., in the case of a FGF-2 polypeptide binding to a naturally occurring ligand, e.g., an FGFR-1. (The invention also includes mimetics of an FGFR-1 peptide which block binding of FGFR-1 to FGF-2.) The critical residues of a subject FGF-2 polypeptide which are involved in molecular recognition of an FGFR-1 polypeptide, can be determined and used to generate FGF-2-derived peptidomimetics which competitively or noncompetitively inhibit binding of the FGF-2 with an FGFR-1 polypeptide (see, for example, "Peptide inhibitors of human papillomavirus protein binding to retinoblastoma gene protein" European patent applications EP-412,762A and EP-B31,080A). For example, scanning mutagenesis can be used to map the amino acid residues of a particular FGF-2 polypeptide involved in binding an FGFR-1 polypeptide, peptidomimetic compounds (e.g. diazepine or isoquinoline derivatives) can be generated which mimic those residues in binding to an FGFR-1 polypeptide, and which therefore can inhibit binding of an FGF-2 polypeptide to an FGFR-1 polypeptide, and thereby interfere with the function of FGF-2 or FGFR-1. Non-hydrolyzable peptide analogs of critical residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gama lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β-turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β-aminoalcohols

(Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Drug Screening Assays

By discovering that an epidermal-dermal interaction can be modulated by modulating, e.g., promoting or inhibiting the activity of FGF-2, the present invention provides assays which can be used to screen for drugs which are either agonists or antagonists of the normal cellular function, in this case, of the FGF-2 polypeptides, or of their role in epidermal-dermal interactions. In one embodiment, the assay evaluates the ability of a compound to modulate binding between an FGF-2 polypeptide and a naturally occurring ligand, e.g., an FGFR-1. A variety of assay formats will suffice and, in light of the present inventions, will be comprehended by skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with other proteins or change in enzymatic properties of the molecular target.

Peptide Analogs of FGF-2

Peptide analogs of an FGF-2 polypeptide are preferably less than 150, 130, 110, 90, 70 amino acids in length, preferably less than 50 amino acids in length, most preferably less than 30, 20 or 10 amino acids in length. In preferred embodiments, the peptide analogs of an FGF-2 polypeptide are at least about 10, 20, 30, 50 or 100 amino acids in length.

Peptide analogs of an FGF-2 polypeptide have preferably at least about 50%, 60%, 80%, 85%, 90%, 95% or 99% homology or sequence similarity with the naturally occurring FGF-2 polypeptide.

Peptide analogs of an FGF-2 polypeptide differ from the naturally occurring FGF-2 polypeptide by at least 1, 2, 5, 10 or 20 amino acid residues; preferably, however, they differ in less than 15, 10 or 5 amino acid residues from the naturally occurring FGF-2 polypeptide.

Useful analogs of an FGF-2 polypeptide can be agonists or antagonists. Antagonists of an FGF-2 polypeptide can be molecules which bind the receptor, e.g., the FGFR-1 receptor, but which lack some additional biological activity such as signal transduction. Antagonists can inhibit an epidermal-dermal interaction, or a process or product that results from an epidermal-dermal interaction, e.g., placode formation, dermal condensation formation, FGFR-1 induction, or the development of a cutaneous appendage, e.g., a hair, a scale, or a feather. Agonists are derivatives which can promote an epidermal-dermal interaction, or a process or product that results from an epidermal-dermal interaction, e.g., placode formation, dermal condensation formation, FGFR-1 induction, or the development of a cutaneous appendage, e.g., a hair, a scale, or a feather.

A large number of analogs of FGF-2 is known in the art. This body of knowledge provides guidance for one skilled in the art to make new FGF-2 analogs.

Using site directed mutagenesis, each of the four cysteines present at amino acid residues 26, 70, 88, and 93 of the mature protein of human fibroblast growth factor 2 (FGF-2) was individually changed to serine (Seno et al., "Stabilizing Basic Fibroblast Growth Factor Using Protein Engineering," in Biochemical and Biophysical Research Communications, (1988), Vol. 151(2):701-708). The biological activity and heparin binding ability was retained when the serine was substituted for the cysteine residue at either 70 or 88 of the FGF-2 protein. This finding indicates that the cysteines at these positions are not essential for expressing biological activity. The substitution of the residues at these positions, especially at position 88, reduced the heterogeneity recognized as several peaks of FGF-2 eluted from a heparin affinity column, even after oxidation with hydrogen peroxide, suggesting that the cysteines at these positions are exposed to the surface of the molecule to form disulfide bonds that induce heterologous conformations. Furthermore, under acidic conditions, these modified FGF-2s are revealed to be more stable in maintaining their activity.

Structural analogs of human fibroblast growth factor 2 (FGF-2) have been prepared by side-directed mutagenesis of a synthetic FGF-2 gene to examine the effect of amino acid substitutions in the three putative heparin-binding domains on FGF's biological activity. Heath et al. (1991) *Biochemistry* 30:5608-5615, discloses a number of such analogs. After expression in *Escherichia coli*, the mutant proteins were purified to homogeneity by use of heparin-Sepharose chromatography and analyzed for their ability to stimulate DNA synthesis in human foreskin fibroblasts. Recombinant human FGF-2 1-146 and [Ala^{69}, Ser^{87}]FGF-2, an analogue where two of the four cysteines had been replaced by alanine and serine, were equipotent to standard bovine FGF-2.

Substitution of aspartic acid-19 by arginine in the first heparin-binding domain yielded a molecule that stimulated a higher total mitogenic response in fibroblasts as compared to FGF-2. In addition, replacement of either arginine-107 in the second domain or glutamine-123 in the third domain with glutamic acid resulted in compounds that were 2 and 4 times more potent than FGF-2. In contrast, substitution of arginine-107 with isoleucine reduced the activity of the molecule by 100-fold. Combination of domain substitutions to generate the [$Glu^{107,123}$]FGF-2 and [Arg^{19},$Lys^{123,126}$]FGF-2 mutants did not show any additivity of the mutations on biological activity. Alterations in the biological activity of the analogues was dependent on both the site of and the type of modification. Increased positive charge in the first domain and increased negative charge in the second and third domains enhanced biological potency. The altered activities of the derivatives appear to be due in part to charges in the affinity of the analogues for heparin. It was conclude that changes in all three of the putative heparin-binding domains result in altered mitogenic activity and heparin interaction of FGF-2.

Springer et al. (1994) *The Journal of Biological Chemistry* 269:26879-26884, using protein structure-based site-directed mutagenesis of FGF-2, identified two FGFR binding sites on FGF-2 which act in concert to initiate signal transduction. Both FGFR binding surfaces are distinct from the heparin sulfate proteoglycan binding domain. The primary, higher affinity, binding interaction comprises a cluster of solvent exposed hydrophobic amino acids (Tyr-24, Tyr-103, Leu-140, and Met-142), and two polar residues (Arg-44 and Asn-101). The hydrophobic contacts dominate the primary binding interaction and provide ~75% of the binding affinity. The secondary FGFR binding site on FGF-2 has an ~250-fold lower affinity and is composed of amino acids Lys-110, Tyr-111, and Trp-114 in a surface-exposed to type I β-turn (formerly known as the putative receptor binding loop).

Zhu et al. (1995) *The Journal of Biological Chemistry* 37:21869-21874, based on peptide mapping and molecular dynamics calculations of the three-dimensional structure of FGF-2, employed site-directed mutagenesis to investigate the effect of altering residues at positions 107, 109-114, and 96 on FGF-2 on receptor binding affinity. All muteins were cloned and expressed in *Escherichia coli*, purified to homogeneity employing heparin-Sepharose columns, and evaluated for receptor binding affinity. It was found that replacement of residues at positions 107 and 109-114 by alanine or phenylalanine had little effect on receptor binding affinities compared with wild type FGF-2, in agreement with previous evidence that FGF-2 residues 109-114 comprise a low affinity binding site. By contracts, substitution of Glu-96 with alanine yielded a molecule having about 0.1% of the affinity of the wild type FGF-2. The affinity of the

corresponding lysine and glutamate muteins was 0.3 and 10%, respectively, emphasizing the importance of a negative charge at this position. These findings are consistent with the view that residues 106-115 on FGF-2 represent a low affinity binding site on FGF-2. In addition, Glu-96 was identified as a crucial residue for binding to fibroblast growth factor receptor-1.

Therefore, it can be summarized that cyteines at residues 26, 70, 88 and 93 of the mature FGF-2 play a role in formation of disulfide bonds that induce heterologous conformations. Residues Asp-19, Arg-107 and Glu-123 seem to play a role in heparin binding, while residues Tyr-24, Tyr-103, Leu-140, Met-142, Arg-44 and Asn-101 play a role in receptor binding. The residues at positions 107 and 109-114 seem to have little effect on receptor binding affinities of FGF-2, while Glu-96 is a crucial residue for binding to FGFR-1.

Equivalents

Those skilled in the art will be able to recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

What is claimed is:

1. A method of modulating an epidermal-dermal interaction comprising modulating FGF-2 activity in one or both of the dermis and epidermis.

2. The method of claim 1, wherein FGF-2 activity is promoted.

3. The method of claim 1, wherein FGF-2 activity is promoted in the dermis.

4. The method of claim 1, wherein said interaction is chosen from placode formation, dermal condensation formation, FGFR-1 induction, or the development of a cutaneous appendage.

5. The method of claim 1, wherein said interaction is the development of hair.

6. The method of claim 1, wherein FGF-2 activity is promoted by administration of exogenous FGF-2 or a fragment thereof.

7. The method of claim 1, wherein FGF-2 activity is inhibited by administration of an anti-FGF-2 antibody or FGF-2-binding fragment thereof, an FGF-2-binding fragment of the FGF-2 receptor, or an antisense molecule which inhibits FGF-2 expression.

8. The method of claim 1, wherein the method is practiced *in vivo* and the subject is a mammal.

9. The method of claim 1, wherein the method is practiced *in vitro* on a tissue or cell.

10. A method of modulating the development of a cutaneous appendage comprising modulating FGF-2 activity in one or both of the dermis and epidermis.

11. The method of claim 10, wherein the appendage is hair.

12. The method of claim 10, wherein FGF-2 activity is promoted.

13. The method of claim 10, wherein FGF-2 activity is promoted in the dermis.

14. The method of claim 10, wherein said cutaneous appendage is hair.

15. The method of claim 10, wherein FGF-2 activity is promoted by administration of exogenous FGF-2 or a fragment thereof.

16. The method of claim 10, wherein FGF-2 activity is inhibited by administration of an anti-FGF-2 antibody or FGF-2-binding fragment thereof, an FGF-2-binding fragment of the FGF-2 receptor, or an antisense molecule which inhibits FGF-2 expression.

17. The method of claim 10, wherein the method is practiced *in vivo* and the subject is a mammal.

18. The method of claim 10, wherein the method is practiced *in vitro* on a tissue or cell.

19. A method for promoting development of a hair on a tissue or subject, the method including administering a therapeutically effective amount of exogenous FGF-2 or fragment thereof to the tissue or subject.

20. The method of claim 19, wherein FGF-2 activity is promoted.

21. The method of claim 19, wherein FGF-2 activity is promoted in the dermis.

22. The method of claim 19, wherein FGF-2 activity is promoted by administration of exogenous FGF-2 or a fragment thereof.

23. The method of claim 19, wherein the method is practiced *in vivo* and the subject is a mammal.

24. A method of evaluating a compound for the ability to bind an FGF-2 polypeptide and the ability to modulate an FGF-2/FGFR-1 interaction or otherwise modulate FGF-2 activity comprising contacting the compound with the FGF-2 polypeptide; evaluating the ability of the compound to interact with the FGF-2 polypeptide; and applying the evaluated compound to an animal, tissue, or cell to further evaluate its effect on an epidermal-dermal interaction, or a process or product that results from an epidermal-dermal interaction.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06309

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 38/18
US CL :514/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS/USPAT; STN/Medline, HCAPlus
search terms: basic fibroblast growth factor or FGF, FGF-2, hair, feather#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	DU CROS, D. L. Fibroblast Growth Factor Influences the Development and Cycling of Murine Hair Follicles. Developmental Biology. 1993, Volume 156, pages 444-453, especially pages 444-445.	1-6, 8, 10-15, 17, 19-23 ------ 9, 18
X ----- Y	DU CROS, D.L. Fibroblast Growth Factor and Epidermal Growth Factor in Hair Development. Journal of Investigative Dermatology. July 1993. Volume 101, Number 1 (Supplement), pages 106S-113S, especially pages 107S-108S.	1-6, 8, 10-15, 17, 19-23 ------ 9, 18
X	JP 05-43424 A (HAYASHIBARA SEIBUTSU KAGAKU; SANSHO PHARMACEUTICAL COMPANY LTD.) 23 February 1993, see abstract.	1-6, 8, 10-15, 17, 19-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
11 JULY 1997

Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06309

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 06-40858 A (SHISEIDO COMPANY LTD.) 15 February 1994, see abstract.	1-6, 8, 10-15, 17, 19-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06309

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6, 8-15, 17-23, as they read on a method of increasing FGF-2 activity

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06309

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-6, 8-15 and 17-23, as they read on a method of increasing FGF-2 activity by administering an FGF-2 agonist.

Group II, claim(s) 1, 4-5, 7-11, 14 and 16-18, as they read on a method of decreasing FGF-2 activity by administering an FGF-2 antagonist.

Claims 1, 4-5, 8-11, 14 and 17-18 are included in both Groups I and II, because they encompass both methods of increasing and decreasing FGF-2 activity. If only Group I is searched, then these claims will be examined only insofar as they read upon a method of increasing FGF-2 activity; if both Groups I and II are searched, then these claims will be searched for both methods of increasing and decreasing FGF-2 activity.

Group III, claim(s) 24, drawn to a method of determining whether a compound binds to FGF-2 and modulate an FGF-2/FGFR-1 interaction.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I is a method of increasing FGF-2 activity by administering FGF-2 or a fragment thereof to an animal. This special technical feature is not shared by Group II because the special technical feature of Group II is a method of decreasing FGF-2 activity by administering an FGF-2 antagonist. Furthermore, neither the special technical feature of Group I nor the special technical feature of Group II is shared by Group III because the special technical feature of Group III is a diagnostic method of determining whether a compound binds to FGF-2. Thus, the three methods do not share a common special technical feature with each other because each method of each group utilizes different product and process steps from one another, and no one method is required for the practice of any other.

Group II contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

1) Anti-FGF-2 antibody
2) FGF-2 binding fragment of the FGF-2 receptor
3) Antisense molecule which inhibits FGF-2 expression

The claims are deemed to correspond to the species listed above in the following manner:

Anti-FGF-2 antibody: 1, 4-5, 7-11, 14, 16-18
FGF-2 receptor fragment: 1, 4-5, 7-11, 14, 16-18
Antisense molecule: 1, 4-5, 7-11, 14, 16-18

The following claims are generic:

1, 4-5, 7-11, 14, 16-18

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

The three species of FGF-2 antagonists are structurally and functionally distinct from one another and do not share a common special technical feature. Although both the antibody and the FGF-2 receptor fragment are proteins which bind to FGF-2, the amino acid sequences of the two proteins are distinct from one another and exhibit different functional characteristics from one another. The antisense molecule is a nucleic acid which is completely different type of molecule than the antibody and FGF-2 receptor proteins, and functions in a completely different manner from the

antibody or receptor proteins. Thus, the three species of FGF-2 antagonists do not share a common special technical feature with one another.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.